Procedure for Confirmatory test on Bovine Spongiform Encephalopathy (BSE) in Prefectures, etc.

1. Institute for test implementation
   (1) Inspection and Safety Division specifies the testing institutes of prefectures, etc. that satisfy the requirements in (2) as "BSE confirmatory test institutes."
   (2) Requirements for specification
      a) Any person who has undergone the technical courses in relation to the BSE confirmatory test provided by the Inspection and Safety Division or any person who is approved by the Inspection and Safety Division as having equivalent or better competence and belongs to the institute.
      b) Necessary devices, etc. indicated in the procedure for this confirmatory test are prepared.
      c) The test method in 2 is observed.
      d) The test technique will be checked as in external precision control notified separately.
2. Implementation of confirmatory test
   (1) For western blot method, the test should be implemented according to Appendix 2-1 "Procedure for immunobiochemical test (western blot method)" and the confirmatory test to be conducted in the prefecture, etc. should be implemented only once.

   Furthermore, if satisfactory test results cannot be obtained, the specimen should be set to the National Institute of Infectious Diseases, etc. for the confirmatory test.

   When sending a specimen, the stored specimen frozen as material for the immunobiochemical test (ELISA method and western blot method) and any remaining sample from the immunobiochemical test in a frozen condition should be sent according to the method of sending a specimen for the confirmatory test.
   (2) For the immunohistochemical test and the histological test, the test should be conducted by the prefecture, etc. according to Appendix 2-2 "Procedure for immunohistochemical test," and the specimen should be sent to the National Institute of Infectious Diseases, etc. for the confirmatory test.

   In addition, remains from the section to be cut out A (A*) – C (C*) illustrated in Figure 1 of Appendix 2-2 Procedure for immunohistochemical test should be included as all sections to be sent in a 50 ml container filled with formalin buffer at room temperature.

(Appendix 2-1)

Procedure for Immunobiochemical Test (Western Blot Method)

1. Instruments, etc.

- Electrophoresis bath: 1 set of XCell SureLock Mini-Cell (Invitrogen EI0001)
- Blotting bath: Mini Trans-Blot Cell (Bio-Rad, 170-3930)
- Power supply: Power Pack 200 (Bio-Rad, 165-5052)
  PowerEase 500 power Supply (Invitrogen EI8600)
- Membrane roller: Membrane roller (Advantech, EBA-200)
- Ultrasonic generator: One with output 750 W or larger or with equivalent output by booster effect (Ex.: Digital sonifier S450D by Bramson)
- Multi-Beads Shocker: Original product by Yasui Kikai Corporation
- Constant-temperature bath (water bath): A water bath that can be used at 37• • (cooling function desired)
- Balance: With minimum weighing unit of 10 mg or smaller
- High-speed refrigerated microcentrifuge: One that is operable at 15,000 rpm or higher

2. Reagents, etc.

- Collagenase (for cell dispersion grade)	Wako	100 mg, No.038-10531
- Pefablock	Roche	500 mg, No.1585916
- Proteinace K, PCR grade	Roche	5 ml, No.1964372
- Dnase I	Roche	100 mg, No.104159
- N-Lauroylsarcosine (Sarkosyl)	Sigma	100 g, No.L-5125
- Zwittergent 3-14	Calbiochem	5 g, No.693017
- Sodium dodecyl sulfate (SDS)	Sigma	500 g, No.L-4509
- 2-mercaptoethanol	Sigma	100 ml, M-6250
- Urea (special reagent class)	Wako	500 g, 217-00615
- 2-Butanol	Wako	500 ml, 020-11215
- Tween 20	Wako	500 ml, 167-11515
- Skim milk	COOP, Meiji, Yukijirushi, etc.	
- Fetal bovine serum (FBS)	Any manufacturer	
- Immobilon-PVDF	Millipore	No.IPVH00010
- Filter paper	Bio-Rad	7.5 x 10 cm, No.170-3932
	ADVANTEC	60 x 60 cm, No.514A

- X-ray film	Fuji Film	6-cut, No.03D051
- ECL western blotting detection reagent	Amersham Pharmacia	No.RPN2209
- Anti-rabbit IgG HRP conjugated	Amersham Pharmacia	1ml, NA9340
- Anti-mouse IgG HRP conjugated	Amersham Pharmacia	1ml, NA9310
- 2ml tube with O-ring	Asyst	No.72.693S

(This is not a tube for Multi-Beads Shocker.)

3. Preparation of reagents

-TN buffer: 100 mM NaCl, 50 mM Tris-HCl (pH 7.5)

- Detergent buffer: 4% Zwittergent 3-14, 1% Sarkosyl,
100 mM NaCl, 50 mM Tris-HCl (pH 7.5)

- Butanol-Methanol mixture: 2-Butanol:Methanol = 5:1(v/v)

-Proteinase K: 1 mg/ml in 50 mM Tris-HCl (pH 8.0), 1 mM CaCl2,
Stored at -20• •by dispensing.

-Pefablock: 0.1M in DDW, stored at -20• •by dispensing.

- Collagenase: 20 mg/ml in DDW, stored at -20• •by dispensing.

- DNase 1: At 10 mg/ml concentration, 50% glycerol, 10 mM Tris-HCl (pH 7.5)
Stored at -20• •by dissolving in 10 mM MgCl2.

- Sample buffer (x 1): 62.5 mM Tris-HCl (pH 6.8), 5% glycerol, 3 mM EDTA, 5% SDS,
4M Urea, 4% ßβ-mercapthoethanol, 0.04% bromo phenol blue,
A small amount in use can be stored at room temperature.
A temperature of 4• •is recommended for long-term storage (Although Urea and SDS may precipitate, they can be dissolved by heating to about 50• •before use).

1 M Tris-HCl (pH 6.8)	1.25 ml
Glycerol	1 ml
0.5M EDTA (pH 8.0)	120µl
• •Mercapthoethanol	800µl
1% bromo phenol blue	800µl
SDS	1 g
Urea	4.8 g
	Up to 20 ml

4. Preparation of brainhomogenate

a) When using the Multi-Beads Shocker (by Yasui Kikai),

1) Insert the metal cone (No.MC-01212PP) in the special tube with 2 ml O-ring.
2) Put 200 mg of the brain tissue into the tube.
3) Add 800μl of TN buffer.
4) Shake for 30 seconds with the Multi-Beads Shocker at 2000 rpm.
5) This is used as 20% (W/W) brain homogenate and it is stored in a tube with O-ring.

b) When using an sonicator,
1) Mince 200 mg brain tissue on Parafilm and transfer to a 2 ml tube.
2) Add 800μl of TN buffer.
3) Processwith ultrasonic using a cup horn-type sonicatoruntil the tissue become a uniform homogenate.
4) This is used as 20% (W/W) brain homogenate and it is stored in a tube with O-ring.

c) When using Enfer method Stomacher homogenizer,
1) Insert 500• 40mg of brain tissue into the homogenizer bag.
2) Add 7.5 ml of Enfer kit Reagent 1 (Enfer Buffer 1 (Bovine)).
3) Process this with Stomacher homogenizer for 2 minutes at speed High.
4) This is used as 6.25% (W/W) brain homogenate and it is stored with division into small volume of 1 ml.

5. Preparation of specimen

1) Add 250μl of Detergent buffer to the 250μL of 20% (W/W) brain homogenate in a 2 ml tube with O-ring and provide vortex (combine with ultrasonic processing if necessary) (Note 1).
2) Add μl of 20 mg/ml collagenase and vortex.
3) Digest for 30 minutes at 37• •(be sure to conduct this in water bath).
4) Add 20μl of 1 mg/ml PK and vortex.
5) Digest for 30 minutes at 37• •(be sure to conduct this in water bath and provide vortex once of twice during digestion).
6) Add 10μl of 0.1M Pefablock and vortex.
7) Add 2μl of 10mg/ml DNase and vortex then left to stand for 5 minutes at room temperature.
8) Add 250μl Butanol-Methanol mixture then vortex.
9) Centrifuge at 15000rpm for 10 minutes at 20• •
10) Remove the supernatant and air dry the precipitate briefly(Note 2).
11) Add 100μl of 1x Sample buffer and boil for 5 minutes at 100• •

If precipitate does not dissolve easily, process with sonication.

a) Preparation of 20% brain homogenate prepared with BSE purification kit (Bio-Rad)

1) Add 250µl of detergent buffer to 250µl of 20% brain homogenate and vortex and ultrasonic processing (Note 1).
2) Add 12.5µl of 20 mg/ml collagenase then vortex.
3) Digest for 30 minutes at 37• •
4) Add 20µl of 1 mg/ml PK then vortex.
5) Digest for 30 minutes at 37• •
6) Add 10µl of Pefablock then vortex.
7) Add 250µ••of Butanol-Methanol mixture.
8) Provide vortex.
9) Centrifuge at 15,000rpm for 10 minutes at 20• •
10) Remove the supernatant and air dry the precipitate (Note 2).
11) Add 100µl 1x Sample buffer and boil for 5 minutes at 100• •

If precipitate does not dissolve easily, process with sonication.

(Note 1) Digestion of non-specific proteins by proteinase K is facilitated and western blot result will be more clear if 25µl (5%) 2-butanol is added to the reaction mixture.

(Note 2) Since supernatant contains butanol, it must be treated as an organic solvent. Add 10 N NaOH of 1/10 volume for prion deactivation and let it stand for 2 hours or longer and then neutralize.

b) Preparation of samples from the 6.25% brain homogenate prepared by Stomacher homogenizer (Enfer method)

1) Centrifuge 1 ml brain homogenate for 10 minutes at 20• •and 15,000 rpm and transfer 800 ml of the supernatant (equivalent to 50 mg tissue) to another 2 ml tube.
2) Add 20µl of 20 mg/ml collagenase then vortex.
3) Digest for 30 minutes at 37• •
4) Add 20µl of 19.2 mg/ml PK for vortex.
5) Digest for 30 minutes at 37• •
6) Add 16µl of 0.1 M Pefablock for vortex.
7) Add 3.4µl of 10 mg/ml DNase then vortex and let it stand for 5 minutes at room temperature.
8) Add 400µl 2-Butanol solution then vortex.
9) Centrifuge at 15,000rpm for 10 minutes at 20• •

10) Remove the supernatant and turn the sample tube over onto a paper towel and let it stand for about 5 minutes to dry the precipitate.

11) Add 100µl 1x Sample buffer and boil for 5 minutes at 100• •. If the precipitate does not dissolve easily, process with sonication.

• •Decontamination of equipment, etc. used in sample preparation

- Scissors, forcepts, chips, tubes, etc. in a pressure-resistant and heat-resistant container for autoclave for 30 minutes at 135• •. In this case, add about 150ml water in the container and do not close the lid.
- Decontaminate inflammables by autoclaving as well.

6. SDS-PAGE

- Use the precast gel by Invitrogen (former Novex).
- Gel: NuPAGE 12% Bis-Tris Gel, 1.0 mm, 12 well (Invitrogen, No.NP0342)
- Use the gel loading chip (Funakoshi SRPT-1381) to load 20µl (equivalent to 10 mg tissue) or 5µl (equivalent to 2.5 mg tissue).
- Buffer: NuPAGE MOPS SDS Running buffer (Invitrogen No.NP0001). Add 500µl Antioxidant (Invitrogen No.NP0005) to the cathode side buffer.
- Conduct electrophoresis at constant voltage of 200 V.

<Positive control for sensitivity measurement>

Use 10-fold dilution of positive control (MoPrP^{Sc} lot 011209, equivalent to 10 mg/ml tissue) with the sample buffer as the original solution (4^0, equivalent to 100 ug/10µl tissue). Heat the original solution (4^0) at 100• •for 2 minutes once when it is prepared. Store the original solution at -20• •by dispensing to about 50µl/tube. Then prepare 4 serial dilution of 4^{-1} (25 ug/10µl), 4^{-2} (6.25 ug/10µl), 4^{-3} (1.6 ug/10µl), and 4^{-4} (0.4 ug/10µl) and store them at -20• •by dispensing. Dissolve the samples at 50• •before use. Load the $4^{-1}$to 4^{-4} positive controls at 10µl/lane (4^{-2} to 4^{-4} is also acceptable depending on the conditions of lane use). Since these positive control dilution lanes are required for WB sensitivity evaluation, conduct electrophoresis on the same gel as the sample. If PrP^{Sc} is detected up to 4^{-3}, the result can be evaluated.

Positive control is distributed by Prof. Horiuchi of Hokkaido Univ. etc.

7. Western Blot (WB)

- Blotting cell: Bio-Rad, Mini Trans-Blot Cell (170-3930)
- Transfer buffer

NuPAGE transfer buffer (Invitrogen No.NP0006)	50 ml	
Antioxidant (Invitrogen No.NP0005)	1 ml	
Methanol	200 ml	final 20%
20% SDS	0.5 ml	final 0.01%
	Up to 1 L	

- Cut a PVDF membrane (Immobilon-PVDF) to 7.5 x 9 cm and soak in methanol for 1 minute to activate it. Then wash with DDW and soak it in the transfer buffer.
- Soak the gel for which electrophoresis has been completed in the transfer buffer.
- Place the PVDF membrane on 2 pieces of filter papers soaked in transfer buffer (1 piece if Bio-Rad filter paper is used) and place the gel on it. Use caution not to let air bubbles form between the gel and PVDF membrane. Then place 2 pieces of filter paper soaked in transfer buffer on the gel.
- Insert the above filter paper-PVDF-gel-filter paper sandwich between blotting pads and set it on the blotting system. Since protein moves from cathode to anode, set it so that the PVDF membrane is at the anode side of the gel.
- Conduct blotting under the conditions of a) – c). a) or b) is recommended. If it must be conducted in haste, c) is acceptable but has a tendency to have high background.
  a) 6 – 15 hours at constant voltage of 30 V
  b) 2 hours at constant voltage of 60 V
  c) 1 hour at constant voltage of 80 V

8. Immunostaining

It is desired that 44B1 is used as the primary method and B103 as the secondary.

[1] When using 44B1 monoclonal antibody (Note 3) (Note 4),

1) Blocking: 5% skim milk, 5% FBS in PBST (0.1% Tween 20). Be sure to heat and dissolve skim milk (at about 80•). After cooling, add FBS was added to the final concentration of 5% (Since 44B1 has a tendency to have high background for PVDF membrane, FBS is added to provide a blocking effect.)
2) Incubate for 1 hour on membrane roller (Advantec, No.EBA-200).
3) Primary antibody: Dilute with 1% skim milk, 1% FBS in PBST. Guideline for concentration of the antibody is 0.1 – 0.2 μg/ml.
4) 1 hour on membrane roller.
5) Wash with PBST for 20 minutes. Replace with PBST 5 times.
6) Secondary antibody (Amersham NA9310): Dilute to 1:2,500 with 1% skim milk

and 1% FBS in PBST.

7) Incubate for 45 minutes on membrane roller.

8) Wash with PBST for 20 minutes. Replace with PBST 5 times.

9) Provide luminescence with ECL western blotting detection reagent.

10) Expose to X-ray film for 2 minutes and develop the film.

11) Expose to another X-ray film while developing.

12) Develop after 30 minutes (prepare X-ray films with 2-minute and 30-minute exposures) (Note 5).

13) Then expose overnight if necessary.

Developing solution: Hi Rendol

Stop solution: 3% acetic acid

Fixing solution: Super Fuji Fix

[2] When using B103 affinity-purified polyclonal antibody (Note 6),

(Differs from [1] 44B1 in 1), 3) and 6).)

1) Blocking: 5% skim milk in PBST (0.1% Tween 20). Be sure to heat and dissolve Skim milk (at about 80•).

2) Incubate for 1 hour on membrane roller (Advantec, No.EBA-200).

3) Primary antibody: Dilute with 1% skim milk in PBST. Guideline for concentration of B103 is 1 μg/ml.

4) Incubate for 1 hour on membrane roller.

5) Wash with PBST for 20 minutes. Replace with PBST 5 times.

6) Secondary antibody (Amersham NA9340): Dilute to 1:2,500 with 1% skim milk.

7) Incbate for 45 minutes on membrane roller.

8) Wash with PBST for 20 minutes. Replace with PBST 5 times.

9) Provide luminescence with ECL western blotting detection reagent.

10) Expose to X-ray film for 2 minutes and develop the film.

11) Expose to another X-ray film while developing.

12) Develop after 30 minutes (prepare X-ray films with 2-minute and 30-minute exposures) (Note 5).

13) Then expose overnight if necessary.

Developing solution: Hi Rendol

Stop solution: 3% acetic acid

Fixing solution: Super Fuji Fix

(Note 3) 44B1 antibody is distributed by Prof. Horiuchi of Hokkaido University, etc. The current lot is 02011, 6.5 mg/ml.

(Note 4) If the background for PVDF membrane is high in the above procedure and specified sensitivity (see p.18) cannot be obtained, it can be improved by the following method:

- •Change the blocking solution and antibody reaction solution to 5% skim milk in 50 mM Tris-HCl (0.1% Tween 20) and 1% skim milk in 50 mM Tris-HCl (0.1% Tween 20), respectively.
- •Wash the membrane after antibody reaction with 0.1% Tween 20 in PBS (50ml) for 5 minutes x 6 times.
- •Use Invitrogen transfer system (Excel II blot module E19051), transfer buffer (NuPAGE transfer buffer: NP0006, NP006-1) and PVDF membrane (LC2005) to transfer for 1 hour at 20 V.

(Note 5) 30 minutes is only a guideline, and it shall be modified flexibly depending on the results of 2-minute exposure.

(Note 6) B103 antibody is available from Fujirebio, Inc. The current lot is SB21103, 1 mg/ml.

Reference: Example of test flow

[Ex.1] Day 1: Preparation of specimen (2 hours)
Day 2: PAGE (1.5 hours) • •WB (2 hours) • •Staining (4 hours)

[Ex.2] Day 1: Preparation of specimen (2 hours) • •PAGE (1.5 hours) • •WB (up to 2 hours)
Day 2: Staining (4 hours)

[Ex.3] Day 1: Preparation of specimen (2 hours) • •PAGE (1.5 hours) • •WB (2 hours) • •Staining (4 hours)

9. Precision control

1) Internal precision control should be implemented at the frequency of every month using the positive control distributed by Prof. Horiuchi of Hokkaido University, etc. and the samples confirmed as negative in the screening test.

2) External precision control should be implemented as notified separately.

3. Firm diagnosis

1) The institutes implementing the confirmatory tests should send the confirmatory test data to the Inspection and Safety Division by electronic media. Furthermore, when conducting confirmatory tests at the prefecture, etc., the corresponding prefecture, etc. should send the confirmatory test data to the Inspection and Safety

Division by electronic media.

2) The Inspection and Safety Division should send the confirmatory test data to the members of the "Expert Committie for BSE Diagnosis" for firm diagnosis.

3) If necessary, obtain a specialist's diagnosis of the histopathological sample (staining sample) using microscopy.

4) The results of firm diagnosis should be informed to the prefecture, etc. implementing the confirmatory test from the Inspection and Safety Division.

(Appendix 2-2)

Procedure for Histology and Immunohistochemistry

1. Preparation of paraffin block

<Preparation>

Disposable bench sheet (laboratory sheet), cutting board, blade for cutting, tweezers, stainless tray, numbered plastic cassettes in the required quantity, can be used for blade disposal, Kimtowel, 1N NaOH, container for formic acid treatment

<Procedure>

1) Cut out the formalin-fixed tissue in the safety cabinet whilst wearing the specified clothing (conforms to the clothing for the screening test).
2) Wipe the outer surface of the specimen container with 1N NaOH and then wipe with a cloth soaked in water.
3) Cut out the formalin-fixed tissue.

Spread a laboratory sheet and place a plastic cutting board, then cut out the tissue on the plastic cutting board with a disposable blade. The thickness for the cut tissue in the obex should be 3 mm or smaller. Cut out the obex first and more 2 pieces from the above to make a total of 3 pieces and place them into a plastic cassette.

For sufficient fixation, shake in 15 – 20% formalin solution for at least 1 hour at 60• •(for rather unfixed material , 2 – 3 hours for fixation are desired). When processing for overnight, fix at 37• •for 1 hour and 30 minutes before the embedding process.

4) Treat with formic acid for 1 hour at room temperature.

Drop the fixed brain tissue in the plastic cassette directly in 98% formic acid solution and shake for 1 hour in a shaker at room temperature. Wash with running water for 30 minutes (to lower infectiousness).

5) Process with sealed automatic embedding system in a 4.5-hour protocol or by manual rotation.
6) Use the system and mold for paraffin embedding which are limited for use of BSE samples .

<Post-processing>

1) Discard formalin in a special waste liquid tank. Incinerate later.
2) Tweezers and blades used in cutting should be placed on the stainless tray to soak in 1N NaOH for 2 hours at room temperature (or place in special can for autoclaving later). Then wash with water. Discard the blades.
3) Cover the cutting board with Kimtowel soaked in 1N NaOH and leave it for 2 hours and then wash with water.
4) Return the remaining brain tissues to the fixing bottle for storage. When they are no longer necessary, put them on autoclave processing (see below) and then discard.

2. Section preparation

<Preparation>

Bench sheet, microtome, bucket of water, paraffin stretcher, humidifier, can for replaced blade disposal, slide glass (silane-coated slide), 1N NaOH, Kimtowel

<Procedure>

1) Wear gloves, shielded mask, and gown. If necessary, use the anti-cut gloves.
2) Spread the bench sheet and place microtome. Prepare a paraffin stretcher and a bucket of water for BSE samples and mount the section on the silane-coated slide.
3) Aspirate the remaining pieces during section preparation with a special vacuum equipped with HEPA filter. Incinerate or autoclave later.
4) Autoclave the knife holder for 60 minutes at 135• •and then wash with water and dry.
5) Soak the blades in 1N NaOH at room temperature (or place in a special can and autoclave later).
6) Dry the sections completely at 45• •

<Post-processing>

Aspirate all remaining paraffin pieces using the previously described vacuum.

3. HE staining (Prepare a special series of Copring jar (container) for paraffin removal and staining)

1) Deparafinization in xylen, through graded ethanol, and immerse in DDW.
2) Harris' hematoxylin staining for 2 minutes at room temperature.

3) Place the sections in warm water for 10 minutes to get clear color.
4) Eosin staining for 3 minutes at room temperature.
5) Clarification, dehydration and clearing in xylene.
6) Mount with a cover glass.

4. Immunohistochemistry

<Reagents>

Envision +kit (DAKO, for mouse and rabbit antibodies),
Simple stain DAB solution (Histofine), 3% hydrogen peroxide in water
Primary antibody to prion, Hematoxyline, PBS

Preparation of PB (0.1M PB for immunity)

$Na_2HPO_4 \cdot 12H_2O$	28.7 g
$Na_2HPO_4 \cdot 2H_2O$	3.3 g
D.W. (distilled water)	1.0 L

Preparation of PBS (0.01M PBS)

PB	100 ml
D.W. (distilled water)	900 ml
NaCl	8.5 g

<Procedure>

1) Deparafinization in xylene, through graded ethanol, and immerse in DDW.
2) Place the sections in distilled water in stainless vat, and autoclave with the specified autoclave for 20-minute at 121 . Transfer the sections in PBS when temperature decreases.
3) Blocking endogenous peroxidase activity by 3% hydrogen peroxide in DDW for 5 minutes at room temperature.
4) Blocking with 10% normal goat serum in PBS for 5 minutes at room temperature (omission possible).
5) Apply primary antibody (described later) and react for 30 – 40 minutes.
6) Wash with PBS.
7) After reaction in Envision+ solution for 30 minutes at room temperature, wash with PBS.
8) Conduct DAB coloring reaction.

9) After washing with tap water and nuclear staining with Mayer's hematoxylin for 30 seconds at room temperature.

10) Clearing in warm water, dehydration, clearing in xylene and mount.

<Post-processing>

Xylene for paraffin removal, ethanol and distilled water shall be discarded in separate special containers to be incinerated.

Process the special staining vat and basket at 135• •for 1 hour and then wash with water.

Discard the staining solutions such as hematoxylin and eosin.

5. Precision control

1) Implement internal precision control at the frequency of every month by using the positive control distributed from the National Institute of Infectious Diseases, etc. and samples confirmed as negative in screening test.

2) Implement the external precision control as notified separately.

1. Illustration of paraffin block preparation (1)
: From embedding (manual)

After cutting, re-fixing by soaking and shaking in 20% formalin for 60 minutes at 60• •

Soaking for 60 minutes in 98% formic acid

Washing in running water for 30 minutes

Removal of excess water with filter paper

80% Ethanol 15 minutes
90% Ethanol 15 minutes
100% Ethanol 15 minutes
100% Ethanol: Acetone
(1:1) 15 minutes
Acetone 15 minutes
Xylene 15 minutes
Xylene 15 minutes
Xylene 15 minutes
Paraffin 15 minutes
Paraffin 15 minutes
Paraffin 15 minutes
Embedding

Required period: 2 hours and 45 minutes

(However, cells tends to be shrinkage.)

1. Illustration of paraffin block preparation (2)

: Processing with automatic embedding system

System used: Sakura automatic sealed fixing embedding system

	Set period
80% Ethanol	10 minutes
90% Ethanol	10 minutes
95% Ethanol	10 minutes
99% Ethanol	20 minutes
100% Ethanol I	20 minutes
100% Ethanol II	30 minutes
100% Ethanol III	30 minutes
Xylene I	20 minutes
Xylene II	20 minutes
Xylene III	20 minutes
Paraffin I	10 minutes
Paraffin II	10 minutes
Paraffin III	10 minutes
Paraffin IV	20 minutes
Total	4 hours and 30 minutes

Vacuum shall be kept ON continuously.

4. Illustration of immunohistochemistry

: Procedure for prompt immunostaining for confirmatory test

Deparaffinization for 10 minutes

Washing in water 5 minutes

Autoclave processing 20 minutes at 121• •

(Soak in distilled water) (Required time 1.5 hours)

Applying 3% hydrogen peroxide water 5 minutes

Washing with PBS 5 minutes x 2 – 3 times

Reaction of primary antibody* 30 – 40 minutes at room temperature

Washing with PBS 5 minutes x 3 times

Reaction of secondary antibody** 30 minutes at room temperature

Washing with PBS 5 minutes x 2 – 3 times

Coloring with DAB 7 – 10 minutes

Washing with running water 5 minutes

Nucleus staining with hematoxylin 30 seconds

Washing with running water (warm water) 5 minutes

Dehydration/mounting 10 minutes

List of reagents and systems

(General reagents and equipment do not need to be from specific manufacturers as long as they are equivalent.)

	Reagent name	Manufacturer name	Standard	Unit
1	Sodium hydroxide	Wako	197-02125	500 g
2	Formalin (37.5%)	Wako	061-00411	3 L
3	Formic acid (98%)	Wako	066-00466	500 ml
4	Paraffin	Wako	164-13345	500 g
5	Alcohol	Sigma		4 L
6	DAKO PEN	DAKO		1 piece
7	Harris' hematoxylin	Mutoh	2002	500 ml
8	Mayer's hematoxylin	Mutoh	3001	500 ml
9	Eosin	Mutoh		500 ml
10	B103 or 44B1 anti-prion antibody	Fujirebio (44B1 is distributed)		
11	Envision+ kit (for mouse or rabbit)	DAKO		110 ml
12	Simple stain DAB kit	Histofine	415172	1 set
13	Hydrogen peroxide water	Wako	081-04215	500 ml
14	Normal rabbit blood serum	Any manufacturer		
15	Xylene for pathological purposes	Mutoh		15 Kg
16	Mount-quick (mounting medium)	Daido Sangyo		30 cc
17	Sodium dihydrogenphosphate (dihydrate)	Wako	199-02825	500 g
18	Disodium hydrogenphosphate•• 12 H_2O	Wako	196-02835	500 g
19	Sodium chloride	Wako	191-01665	500 g

	Equipment/system name	Manufacturer name	Standard	Unit
1	Disposable bench sheet	Wattmann	40 x 57 cm	50 sheets
2	Disposal blades for sectioning and cutting	Feather	No.130	A set of 50 blades
3	Plastic cassettes	Tissue-Tek	Procassette	1.000 pieces
4	Kimtowel	CRECIA	J-120	24 bundles
5	Container for formic acid processing	NALGENE	2118-0032	pieces
6	Silane-coated slides	Mutoh	1106	100 pieces
7	Cover glass	Mutoh	24 x 36	1,000 pieces
8	Microtome disposal blades	Feather	A35	50 pieces
9	Staining vat (for 20 pieces)	Matsunami		pieces
10	Staining basket (for 20 pieces)	Matsunami	B-20	pieces
11	Stainless vat		0.6L	pieces
12	Humidifying box	COSMO BIO	for 20 pieces	pieces
13	Latex gloves	Asahi Emas	DPG-350	Box (100 gloves)
14	Masks with plastic shield	Hogy Medical	FBM-281	50 pieces
15	Gowns	Hogy Medical	MGM-14	30 sets
16	Anti-cut gloves	Inai	LA132	10 gloves
17	Safety cabinet	Laboconco	LAD-1300XA	
18	Slide washer	Juji Field	SW-4	
19	Autoclave 135• •	Tomy Seiko	KS-323	
20	Hood with HEPA filter	Oriental	Aura-700	
21	Vacuum with HEPA filter	Atomic	FC-111-A13	
22	Automatic embedding system	Sakura Finetek	ETV-150CV	
23	Paraffin stretcher	Sakura Finetek	PS-53	
24	Humidifier	Sakura Finetek	SMB-1	

(Supplementary) Procedure for preprocessing on anti-PrP antibody and pathological pieces

At present, anti-PrP peptide rabbit antibody and mouse monoclonal antibody are available for BSE confirmatory test. The former consists of B103 (Obihiro University of Agriculture and Veterinary Medicine) and T4 (National Institute of Infectious Diseases) and the latter consists of 44B1 and 43C5 (both from Obihiro University of Agriculture and Veterinary Medicine). It was discovered that the combination of preprocessings on antibody and specimen influence the results. As of now, it is desired that B103 is used as the primary antibody and 44B1 as the secondary after autoclaving in distilled water.

1. Preprocessing on sections

Preprocessing on the stissue ections after deparaffinization is conducted for the purpose of enhancement reaction with PrPSC (recovery of antigenicity or unmasking of antigens), and it is an inevitable process for BSE testing. The following 2 types of preprocessing methods were examined:

1) 20 minutes at 121• •in distilled water

2) 20 minutes at 121• •in 1mM HCl solution

Conventionally, proteinase processing was conducted additionally. However, it is not required in the current prompt fixing and embedding method.

For both of the above 2 methods, staining basket containing thesections was placed in a 400 ml stainless vat with a lid for autoclaving under the same conditions.

2. Antibody properties

a) B103 rabbit antibody: Prepared using the 103-121 peptide of PrP protein N-end as the antigen. Conc.4.6 mg/ml

b) T4 rabbit antibody: Prepared using the 221-239 peptide of PrP protein C-end as the antigen. Conc.0.6 mg/ml

• •The above 2 rabbit antibodies are affinity purified antibodies.

c) 44B1 mouse monoclonal antibody: Recognizes 155-231. Conc. 4 mg/ml

d) 43C5 mouse monoclonal antibody: Recognizes 161-169. Conc. 4.6 mg/ml

3. Antibodies and methods of preprocessing

Antibody and dilution scale	1) DDW 20 minutes at 121• •		2) 1mM HCl 20 minutes at 121• •	
	Pos	Neg	Pos	Neg
B103 x500	+/-	-/-	3+< / +N	-/+D
T4 x1000	2+/-	-/-	3+> / -	-/+-
44B1 x500	+/-	-/-	2+< / -	-/-
43C x2000	2+/+D	-/+	3+/2+D	-/3+D

Pos: Positive control (2nd case in Hokkaido), Neg: Negative control (case with nonspecific observation; B026)
+/-: Signal positive (degree) / nonspecific reaction (degree)
The antibodies used here were manufactured by Fujirebio and the antibody concentration was 1 mg/ml.

4. Remarks

1) B103 is a monospecific polyclonal antibody, and it is expected to be used to recognize several antigen-determining groups. It is normally possible to detect PrPSC without problems under the conditions of 1). However, reactivity is rather weak. Weak nonspecific reaction may be observed in cell nuclei. Nonspecific staining of nuclei is especially strong under conditions of 2). Although staining under conditions of 2) is best, it accompanies nonspecific reactions and 1) is recommended as of now.

2) Best results are obtained under the conditions of 2) for T4 antibody. Nonspecific staining was observed in 3 cases in the past. Such nonspecific positive reaction was similar for B103 and 43C5 as well. However, it was not observed for 44B1. Furthermore, T4 does not remain in volume to be distributed.

3) While 44B1 can be used under any condition without nonspecific reactions, it has somewhat weak reaction (signal intensity). The staining intensity is stronger for 43C5 than for 44B1.

4) 43C5 shows nonspecific reactions in which staining is seen diffusively on the nerve cells and net such as olivary nucleus under any method.